The magnesium-insertion step of chlorophyll biosynthesis is a two-stage reaction

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Mg^{2+}-chelatase catalyses the first step unique to chlorophyll synthesis, namely the insertion of Mg^{2+} into protoporphyrin IX. When pea (*Pisum sativum* L., cv. Spring) chloroplasts are lysed in a buffer lacking Mg^{2+} and the thylakoids removed by centrifugation, the remaining mixture of light membranes and soluble proteins (LM/S) has high Mg^{2+}-chelatase activity. Several lines of evidence are presented to show that the Mg^{2+} insertion catalysed by this preparation is a two-step reaction consisting of activation followed by Mg^{2+} chelation. An activated state of Mg^{2+}-chelatase is achieved by preincubating LM/S with ATP. The activated state is observed as the elimination of the approx. 6 min lag in the rate of Mg^{2+} chelation on addition of the porphyrin substrate. The activity of LM/S assayed at low protein concentrations can be greatly enhanced by preincubating at high protein concentrations (12 mg/ml is optimal). This activation effect requires the presence of both LM and S fractions, as well as ATP. Both steps require ATP, but at different concentrations; the first step is optimal at > 0.5 mM (EC_{50} = 0.3 mM) and the second step is optimal at 0.3 mM (EC_{50} < 0.2 mM). ATP in the first step could be replaced by ATP[S]; this analogue could not sustain activity in the second step. This activated state was stable for at least 30 min at room temperature, but chilling of preincubated LM/S on ice for 30 min caused an almost complete loss of the activated state.

INTRODUCTION

Higher plant chloroplasts have a capacity for both haem and chlorophyll biosynthesis. As might be expected from the structural similarities of these two porphyrins, haem and chlorophyll have many enzymic steps in common in their biosynthetic pathways. Currently, it is believed that intermediates up to the level of protoporphyrin IX (Proto) are shared by the two pathways; the pathways diverge after Proto [1]. The 'branch point' thus created is at the key step of metal ion chelation. Insertion of iron into Proto is the first step unique to haem synthesis, whereas insertion of Mg^{2+} into the same substrate is the first committed step in the chlorophyll pathway. The enzymes that catalyse metal-ion insertion are ferrochelatase and Mg^{2+}-chelatase respectively.

As ferrochelatase and Mg^{2+}-chelatase catalyse virtually identical reactions, namely the insertion of a bivalent metal ion into Proto, it might be predicted that their mechanisms would be very similar. However, this does not appear to be the case. Ferrochelatase activity has been measured in chloroplast extracts [2–4] as well as in bacterial [5] and mammalian systems [6]. In the cases where it has been purified, the enzyme always consists of a single membrane-bound protein which uses Proto and reduced iron as its sole physiological substrates. In contrast, Mg^{2+}-chelatase has not been well characterized. Castelfranco and co-workers were able to assay the enzyme in intact cucumber chloroplasts [7–10]. They found that the enzyme required the porphyrin and metal ion for activity, but surprisingly it also had a requirement for ATP. Further characterization of the enzyme was hampered by a complete loss of activity after plastid breakage.

More recently, we were able to demonstrate that high Mg^{2+}-chelatase activity could be measured in extracts of broken pea (*Pisum sativum* L., cv. Spring) chloroplasts [11]. In our system, the enzyme required two protein fractions, one soluble and one membrane-bound. If the chloroplasts were lysed in buffer lacking $MgCl_2$, a highly active fraction resulted when the bulk of the thylakoid membranes were removed with a low-speed spin [12]. The fraction remaining was essentially a mixture of 'light' membranes, probably envelope, and stroma (LM/S). This fraction could be further separated into LM and S by ultracentrifugation; both subfractions are required to reconstitute Mg^{2+}-chelatase. As the LM/S has very little chlorophyll, we were able to develop a continuous fluorimetric assay for the enzyme. In this and subsequent studies, deuteroporphyrin IX (Deutero) was used as the porphyrin substrate instead of Proto, because of its higher aqueous solubility and stability compared with Proto [13].

Using this assay, we described several phenomena. First, the time course of Mg-Deutero accumulation is not linear; a lag of about 5–6 min preceded the appearance of product. Moreover, this lag could be eliminated if the LM/S was preincubated in the presence of ATP. Preincubation in the absence of ATP caused a complete loss of activity. Thus ATP seemed to have the dual roles of activation and stabilization. Secondly, the shape of the enzyme activity versus protein concentration curve was concave up rather than linear as is seen in intact plastids; at low LM/S protein concentrations (0.7 mg/ml) there was very little activity and a linear response was seen only above 1.2 mg/ml LM/S protein.

We suggested that these data could be explained by a model in which the Mg^{2+}-chelatase components must come together and interact in an ATP-dependent activation step before activity is observed. At lower protein concentrations the components are too dilute to 'meet' each other for this activation process to occur. Similarly, even if the components can come together, the

Abbreviations used: Deutero, deuteroporphyrin IX; Proto, protoporphyrin IX; LM, light chloroplast membranes; S, soluble chloroplast proteins; ATP[S], adenosine 5′-*O*-(3-thiotriphosphate); Rubisco, ribulose bisphosphate carboxylase/oxygenase; AMPPCP, adenosine 5′-[β,γ-methylene]-triphosphate.

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activation process does not occur in the absence of ATP. An example of this type of mechanism would be a protein kinase-dependent activation of an otherwise inactive Mg^{2+}-chelatase. Alternatively, a specific ATP-dependent activase may be involved such as has been reported for ribulose bisphosphate carboxylase/oxygenase (Rubisco) [14]. In either case, Mg^{2+}-chelatase activity would have to be a two-step process consisting of activation followed by catalysis. In the present paper we have attempted to demonstrate how the Mg^{2+}-chelatase reaction proceeds, with particular emphasis on confirming the two-step nature of the process.

MATERIALS AND METHODS

Materials

Peas (*Pisum sativum* L., cv. Spring) were purchased from Asgrow seeds (Doraville, GA, U.S.A.). Tricine and dithiothreitol were purchased from Research Organics (Cleveland, OH, U.S.A.). BSA and Miracloth were obtained from Calbiochem (San Diego, CA, U.S.A.). Deutero was purchased from Porphyrin Products (Logan, UT, U.S.A.). All other biochemicals were purchased from Sigma (St. Louis, MO, U.S.A.), and all organic solvents and salts were of analytical grade or better.

Chloroplast isolation

Pea seeds were washed with tap water to remove excess Captan fungicide and then allowed to imbibe water for 1.5–3 h. The peas were grown at about 26–32 °C for 7 days under a 12 h light/12 h dark cycle in trays of moist vermiculite. Seedlings were harvested for chloroplast isolation between 3 and 6 h after the final light cycle started. Intact chloroplasts were isolated as previously described [13].

Chloroplast fractionation

For routine organelle-free Mg^{2+}-chelatase assays, the chloroplasts were lysed and fractionated by a procedure adapted from Keegstra and Yousif [15]. Intact chloroplasts were resuspended in a lysis buffer referred to as TE buffer [10 mM Tricine, 2 mM EDTA, 1 mM dithiothreitol, 0.0025% (w/v) phenylmethanesulphonyl fluoride, pH 7.8] at a protein concentration between 30 and 40 mg/ml. After 10 min on ice, the chloroplast suspension was subjected to two freeze (−20 °C)/thaw cycles of at least 30 min. The lysed plastids were centrifuged at 13000 ***g*** for 2 min in a microcentrifuge maintained at 4 °C, yielding pellet and supernatant fractions. The supernatant was carefully removed and retained, and the green pellet was resuspended in half the original volume of TE buffer and centrifuged again at 13000 ***g*** for 2 min. The supernatant was removed and combined with the first supernatant, and made up to 10 mM $MgCl_2$. The pooled supernatants, consisting mainly of soluble proteins and lighter membranes (referred to as LM/S), could be stored at −70 °C, used directly in Mg^{2+}-chelatase assays or further fractionated as described below. Separation into pellet and soluble fraction was achieved by centrifugation for 30 min at 260000 ***g***. After removal and saving of the soluble fraction (mostly stromal enzymes), the pellet was resuspended in TE buffer containing 10 mM $MgCl_2$, and centrifuged again at 260000 ***g*** for 30 min. This wash was used to minimize contamination of the pellet with soluble proteins; it was routinely discarded. The final washed membrane pellet was resuspended in TE buffer (containing 10 mM $MgCl_2$). Any deviations from the standard chloroplast fractionation are indicated in the Results section.

Mg^{2+}-chelatase assays

In most cases a continuous fluorimetric assay, as described by Walker et al. [12], was used to determine Mg^{2+}-chelatase activity. In cases where LM and S were recombined, a stopped assay was used; this was because of the tendency of isolated LM fractions to sediment during the assay which would adversely effect the continuous assay. The stopped assay was also more convenient when multiple samples required rapid assessment of activity. The stopped assay was performed exactly as previously described and the reaction product, Mg-Deutero, was extracted and quantified fluorimetrically [13]. Both assays contained identical mixtures of cofactors and substrates at the following final concentrations: 9.0 μM Deutero [in dimethyl sulphoxide, 2% (v/v)], 2.0 mM ATP in a regenerating system [10 mM phosphocreatine/creatine kinase (2 units/ml)], 9.0 mM $MgCl_2$, pH 7.8 [12]. In some cases, the samples were preincubated for 6 min at 30 °C in a reaction buffer consisting of all components except the Deutero. The reactions were then initiated by the addition of Deutero. In most cases where ATP was used, the regenerating system was also present; any deviation from these standard procedures are indicated in the Figure legends. As LM/S gradually loses activity over long periods (hours) when stored on ice, LM/S samples to be assayed sequentially with the continuous assay were stored at −70 °C in small portions which were then individually thawed just before each assay. One unit of activity is defined as 1 pmol of Mg-Deutero formed in a 30 min incubation at 30 °C. Tabular data are presented as the average of duplicate measurements ± the deviation from the average; the deviation was rarely greater than 10% of the average.

Other procedures

Chloroplast protein was determined by the method of Bradford using BSA as a standard [16]. Small-volume high-speed centrifugations were performed in a Beckman TL 100 benchtop ultracentrifuge. 6-Phosphogluconate dehydrogenase activity was assayed as a stromal marker by the method of Journet and Douce [17].

RESULTS

Rationale

We are suggesting that Mg^{2+} chelation is a two-step process. In the first step, the components of the Mg^{2+}-chelatase system must interact together, with ATP, in order to achieve activation. In the second step the activated enzyme(s) can catalyse the actual Mg^{2+} insertion into the porphyrin ring. In order to test this hypothesis, we have devised a number of experiments to test specific predictions of a two-stage reaction, in which the first stage is activation. As we know that the overall reaction requires two protein fractions (LM and S), ATP, Mg^{2+} and Deutero, the most obvious place to start is to examine these requirements in the two stages; we would expect these requirements to vary in the two stages if they are qualitatively different. For example, we have observed that a plot of activity of the overall reaction versus LM/S protein concentration is curved (concave up) rather than linear. Although this phenomenon may simply indicate that more than one protein is required for the reaction, it may also be an indication that one protein is activating another. Insight into this possibility can be gained by varying the protein concentration of preincubation and incubation independently.

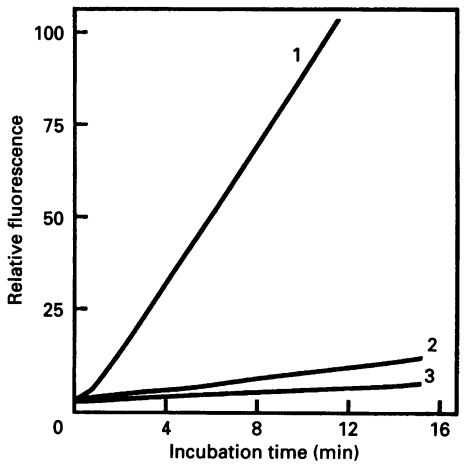

Figure 1 Enhancement of Mg^{2+}-chelatase activity by preincubation at high protein concentrations

Mg^{2+}-chelatase activity was measured by the continuous assay (see the Materials and methods section). All samples were preincubated at 30 °C for 6 min before assay and all samples were assayed at identical protein (1.5 mg/ml) and ATP (2 mM) concentrations. In the control (line 2), the sample was preincubated in the presence of 2 mM ATP and the reaction was initiated by the addition of Deutero. Preincubation and assay were at the same protein concentration. A second sample was preincubated at a 4-fold higher protein concentration (6.0 mg/ml) in the presence of 2 mM ATP (line 1). The reaction was initiated by dilution with buffer (final protein concentration 1.5 mg/ml) and addition of Deutero and ATP (final concentration 2 mM). A third sample was also preincubated at the higher protein concentration (6.0 mg/ml) as described above, except that no ATP was included in the preincubation buffer (line 3). Time zero is defined as the time when all components, including the Deutero, are present. The specific activity for line 1 was 109 units/mg. This activity was derived from the slope of the linear portion of the trace.

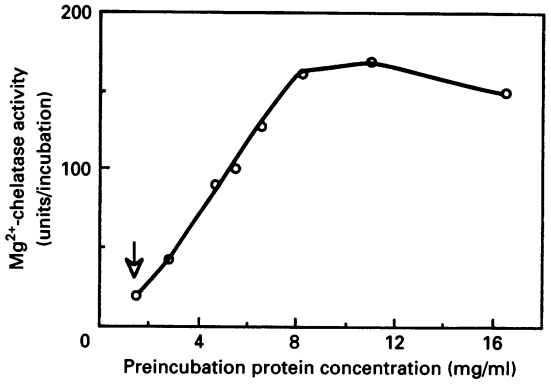

Figure 2 Effect of varying protein concentration during preincubation

Samples were preincubated with various amounts of LM/S protein for 6 min at 30 °C in the presence of 2 mM ATP. At the end of the preincubation period, all samples were diluted to a final protein concentration of 1.5 mg/ml, an ATP concentration of 2 mM, and Deutero was added to initiate the reaction. The arrow indicates the preincubation concentration which is identical with the final assay concentration (i.e. no dilution).

Effect of varying protein concentration during a preincubation

The protein concentration-dependence of the first step can be determined by assaying Mg^{2+}-chelatase in two stages. First, LM/S is preincubated (6 min at 30 °C) in the presence of 2 mM ATP at a high protein concentration and in the absence of the porphyrin substrate; this would permit the activation step to take place [12]. The sample is then diluted to a low protein concentration with buffer containing Deutero and ATP; the composition of the dilution buffer is adjusted to give the desired final concentration of all substrates and LM/S protein during the assay. The production of Mg-Deutero after dilution is monitored fluorimetrically with the continuous assay. With this procedure, Mg^{2+}-chelatase activity was clearly detectable (Figure 1, line 1) at low protein concentrations (see Figure 1 legend). This activity was 12-fold higher than the control (line 2) which was preincubated with ATP at a low protein concentration and assayed at the same lower protein concentration (i.e. the reaction was initiated by the addition of Deutero, but without dilution). Both the activities shown in lines 1 and 2 (Figure 1) were therefore assayed under identical conditions; the only difference was that the high activity sample (line 1) was preincubated at a 4-fold higher protein concentration. Preincubation at a high protein concentration (6 mg/ml) in the absence of ATP results in an almost complete loss of activity (line 3). This stimulation of activity was specific to LM/S protein and could not be reproduced by preincubating LM/S in the presence of 19.0 mg/ml BSA and ATP (results not shown) and assaying after addition of Deutero as detailed above.

Using the same experimental strategy as above, the optimal protein concentration in the preincubation step was determined. Again, all assays were performed at identical final protein and substrate concentrations, but the preincubation protein concentration was varied as indicated (Figure 2). In the control (indicated by the arrow), which was preincubated and assayed at the same low protein concentration, there was low activity. However, Mg^{2+}-chelatase activity increased with increasing protein concentration in the preincubation, up to about 10 mg/ml LM/S protein; protein concentrations above this did not enhance the activity any further. We find that this optimum varies slightly between different preparations.

Protein fractions required for activation

Our system requires two different protein fractions for activity [12], and both of these fractions may contain more than one necessary component for activity. To test whether the activation requires both fractions, LM/S was separated into membrane (LM) and soluble (S) fractions by high-speed centrifugation (Materials and methods section). As a reference point a control of recombined LM and S was assayed with no preincubation (Table 1). The activity of this control represents zero activation; activities above this value indicate activation. The greatest activation was seen when the preincubation step contained both LM and S together; in this case, the activity was almost 3-fold higher than the control. The slight (60%) activation observed when the S fraction was preincubated alone is most likely due to cross-contamination with LM component(s). We have observed that the active component(s) of the LM fraction can be partially removed by washing the membranes with dilute buffer (unpublished results). Thus the S fraction almost always demonstrates some residual activity when assayed at high protein concentration, but in the absence of LM.

Effect of low temperatures on the activation step

Although it is clear that the activation step requires both LM and S fractions, it does not necessarily follow that the catalytic step requires both fractions. To determine whether the activated state would be stable for the centrifugation time required for LM and S separation (30 min, [12]) the following procedure was em-

Table 1 Requirement for both membrane and soluble fractions in the preincubation for activation

LM and S fractions were prepared as described in the Materials and methods section. The fractions (0.3 mg of protein each) were preincubated separately or together at 30 °C for 6 min in 100 μl of assay buffer containing 2 mM ATP (plus regenerating system) and 9 mM $MgCl_2$. At the end of the preincubation period, the other protein fraction was added if not already present, and the mixture diluted with buffer, ATP, $MgCl_2$ and Deutero to 250 μl and the normal substrate concentrations. The activity was measured using the standard stopped assay procedure.

Fraction(s) preincubated	Specific activity (units/mg)	Stimulation caused by preincubation (%)
LM + S, no preincubation (control)	97.0 ± 3	–
LM alone	105 ± 3	8
S alone	155 ± 0	60
LM + S	277 ± 10	186

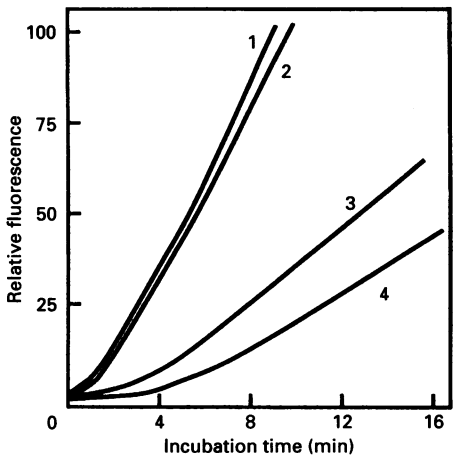

Figure 3 Effect of chilling on Mg^{2+}-chelatase activation

LM/S was preincubated (12 mg/ml protein) with 2 mM ATP for 6 min at 30 °C, then subjected to the following treatments. Line 1, the sample was maintained at room temperature for 30 min before dilution and assay. Line 2, the sample was diluted and assayed immediately. Line 3, the sample was chilled on ice for 30 min before assay. For the assays, all samples were diluted to 2 mg/ml LM/S protein with warm buffer (containing ATP and Deutero to bring their final concentrations to 2 mM and 9 μM respectively). The control, line 4, was assayed directly under conditions identical with the above samples, but without any preincubation. The specific activity of the sample shown in line 4 was 107 units/mg.

ployed: LM/S was preincubated with ATP (6 min at 30 °C), put on ice for 30 min, then diluted with warm buffer and assayed for Mg^{2+}-chelatase activity by the addition of Deutero. The continuous assay was used to determine whether the activity was linear from the addition of porphyrin substrate (as would be expected for an activated sample) or whether there was a substantial lag. The control was tested in an identical manner, except that the sample was assayed immediately after the preincubation period to demonstrate that activation had taken place. The activity trace of the sample which was chilled for 30 min showed a significant lag before Mg^{2+} chelation started (Figure 3, line 3). Virtually no lag was seen in the control trace (line 2), and the rate of Mg^{2+}-chelatase activity in this sample

Table 2 Fractionation of LM/S after preincubation with ATP

In treatment A, LM/S was preincubated (13 mg/ml) at 30 °C for 10 min in the presence of 1 mM ATP (plus regenerating system). The sample was then separated into LM and S fractions by centrifugation for 20 min (260000 *g*) at 25 °C. The LM pellet was resuspended in buffer containing 1 mM ATP (plus regenerating system). The fractions, alone and reconstituted, were assayed immediately for Mg^{2+}-chelatase activity using the stopped assay (see the Materials and methods section). The ATP concentration in the assay was 0.25 nM and the assay time was 10 min. Treatment B was identical with A, except there was no preincubation and the ultracentrifugation was at 4 °C. Treatment C was identical with B except that the assay time was 30 min and the ATP concentration in the assay was 2 mM. The protein amounts in the 200 μl assay were: treatment A, 0.25 mg of LM and 0.18 mg of S; treatment B, 0.25 mg of LM and 0.22 mg of S; treatment C, 0.50 mg of LM and 0.44 mg of S. The assay conditions (time, ATP and protein concentrations) for treatments A and B were designed to minimize activation during the assay. Treatment C was a control to ensure that the fractions were active. 6-Phosphogluconate dehydrogenase activity was used as a marker of S cross-contamination in the LM; in treatment A there was 10.5% cross-contamination and in treatments B and C there was 11.2%.

Fractions(s) assayed	Treatment...	Mg^{2+}-chelatase activity (units/incubation) A	B	C
LM		14.0 ± 0.2	9.6 ± 0.6	25.7 ± 0.9
S		16.8 ± 0.0	0.0 ± 0.0	10.5 ± 0.2
LM + S		59.4 ± 0.3	11.7 ± 1.2	169 ± 7.0

was about 2-fold higher than the sample chilled between preincubation and assay. The chilled sample did have slightly higher (37%) activity than a sample that was assayed without any preincubation (line 4). Although the activated state was not stable on ice, a sample that was left at room temperature for 30 min after preincubation (rather than chilled) did retain its activated state (line 1); the activity trace of this sample was almost identical with the control (line 2), showing high activity and no lag. The inactivation caused by chilling was reversible. If the preincubated chilled sample was rewarmed before dilution and assay, there was complete retention of the activated state (high activity and no lag; results not shown). It should be noted that the effect of chilling seen here was not due to a direct temperature effect on enzyme activity (rather than activated state); the samples were diluted with warm buffer before assay and incubated in a thermostatically controlled cuvette. To further eliminate possible small assay temperature differences, an equal volume of chilled buffer was added to the unchilled sample to compensate for the chilled enzyme. In this separate experiment the cryoinactivation phenomenon was still observed (see Table 3, first column).

Separation of components after activation

To determine whether only one fraction was required for Mg^{2+} chelation after the activation step, LM/S was preincubated for 10 min at 30 °C in the presence of 1 mM ATP (optimal conditions for activation, see below). After preincubation, the LM/S was maintained at 25 °C during the separation into LM and S fractions by ultracentrifugation. These fractions were immediately assayed separately and together (Table 2, treatment A). In an attempt to measure only the activity from already 'activated' protein, the samples were assayed under conditions that would minimize new activation during the assay: this involved assaying for only 10 min at lower protein concentrations and in the presence of only 0.25 mM ATP, which is suboptimal for activation (see below). The data show that LM and S assayed together had at least 250% more activity than either fraction

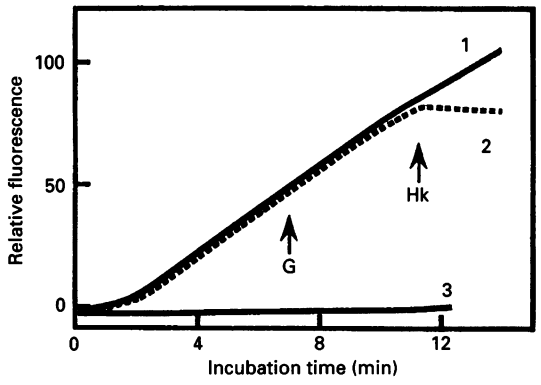

Figure 4 Is ATP required for both steps of Mg^{2+} chelation?

LM/S was preincubated for 6 min at 30 °C at 1 mM ATP, then diluted 5-fold and assayed with 0.2 mM ATP and 9 μM Deutero (1.8 mg/ml LM/S protein). The regenerating system was kept at the standard concentrations in both the preincubation and the assay. Line 1, no ATP trap; line 2, glucose (G) (50 mM final concentration) and hexokinase (Hk) (10 units) were added at the times indicated by arrows; line 3, hexokinase and glucose were added at the beginning of the preincubation. The specific activity of the sample without the glucose/hexokinase ATP trap was 139 units/mg.

alone. Thus it is clear that not all of the activity resides in a single fraction after activation.

Owing to the irreversible loss of Mg^{2+}-chelatase activity at room temperature in the absence of ATP (Figure 1, line 3 and [12]), it was not possible to compare the numbers in Table 2 with a sample treated identically, but in the absence of ATP. Therefore two other types of control were devised. In treatment B, LM/S was separated by ultracentrifugation in the cold without a prior preincubation. The samples were assayed separately and together under the same conditions as in treatment A (i.e conditions to minimize activation during the assay). In treatment C, LM/S was separated in the cold as in treatment B, but assayed under standard conditions (30 min, higher protein concentration and 2 mM ATP). Treatment C demonstrated the usual effect of separation and reconstitution of fractions, namely, low activity in either fraction alone and synergistic activity when they were reconstituted [12]. Comparison of treatments A and B revealed several important properties of the activation. (1) The activity of the combined fractions in treatment A was 5-fold greater than in treatment B, indicating that the activated state survived separation and reconstitution. (2) The activities of the LM fractions were almost identical, indicating that the activity in this fraction had not been affected by the activation step and that, clearly, this fraction did not contain a single activated component. It should be noted that in all three treatments, the LM fractions were contaminated by approximately 11% of the total 6-phosphogluconate dehydrogenase activity which serves as a marker for the soluble components [12,17]. This cross-contamination of the LM with S readily explains the residual activity in the LM. (3) The activity of the S fraction in treatment A was higher than in either of the other two treatments, with the difference being most dramatic between treatments A and B. In treatment A this fraction appeared to be active by itself, although the activity was still synergistically increased by the addition of LM. Unfortunately, in the standard assay we often observed residual activity in the S fraction alone (treatment C) which we attributed to cross-contamination of S with LM (see above). Until we are able to isolate 'clean' fractions and stabilize the enzyme at room temperature in the absence of ATP, it may not be possible to determine unambiguously if a single fraction is activated and catalyses Mg^{2+} chelation in the absence of the other.

ATP requirement for the first and second steps

From our previous data, it is clear that ATP is absolutely required for activation in the first step, but this requirement has not been demonstrated for the second step. To determine whether ATP is in fact needed in the second step, LM/S was preincubated at a high protein concentration (9 mg/ml) with ATP. After preincubation, the sample was diluted 5-fold with buffer containing Deutero but lacking additional ATP. The time course of activity was linear, despite the 5-fold lower ATP concentration in the incubation than in the preincubation (Figure 4, line 1). From these data it seemed possible that ATP might not be required in the second stage. In order to remove all remaining ATP, glucose and hexokinase were added sequentially to act as a trap. In the presence of the trap, Mg^{2+}-chelatase activity immediately stopped (line 2). The order of addition of glucose and hexokinase made no difference to this result (not shown); the presence of the trap during the preincubation period completely abolished activity (line 3).

Although activation and Mg^{2+} chelation both required ATP, it is possible that the ATP was playing a different role or interacting with different proteins in each of the two steps. In this case, the concentrations of ATP needed for the first and second steps could be quite different. The ATP concentration-dependence of the first step was determined as follows. LM/S was preincubated in a small volume in the presence of various concentrations of ATP. After preincubation, each sample was diluted with buffer containing Deutero and the appropriate amount of ATP to bring the final ATP concentration up to 0.5 mM during the subsequent continuous assay. Thus all samples were assayed at identical ATP concentrations, but preincubated at different ATP concentrations as indicated in Figure 5. The EC_{50} for ATP in the preincubation was approx. 0.3 mM, and activity was saturated between 0.5 and 1.0 mM. A similar strategy was used to determine the ATP-dependence of the second step. The preincubations were all carried out at 1.0 mM ATP, then the samples were diluted with buffer containing Deutero and sufficient ATP to bring the final ATP concentration in the assay to the desired amount. Clearly, this experiment limits the lowest concentration that can be tested in the assay (dilution with Deutero and no ATP results in 0.2 mM ATP in the assay). The ATP-dependence of the second step was shifted to lower concentrations compared with the first; at 0.2 mM ATP in the assay, the Mg^{2+}-chelatase activity was only slightly lower than the activity measured at the optimal concentration of about 0.5 mM. It was impossible to obtain an accurate EC_{50} because we could go no lower than 0.2 mM, but clearly the EC_{50} would be less than 0.2 mM. When the ATP concentration was the same in the preincubation and the assay, the activity dependence on ATP concentration was identical with that observed for the preincubation (i.e. the overall reaction was limited by the ATP required in the activation step). Both the preincubation and the overall assay were tested with ATP concentrations up to 2.0 mM. In each case the activity was the same as at 1 mM ATP (results not shown). All preincubations and assays contained the standard ATP-regenerating system, regardless of the concentration of ATP (see Figure 5 legend).

Using the same experimental strategy, the Mg^{2+} concentration requirement for activation and catalysis was investigated. The optimal Mg^{2+} concentration was the same for both steps (results not shown).

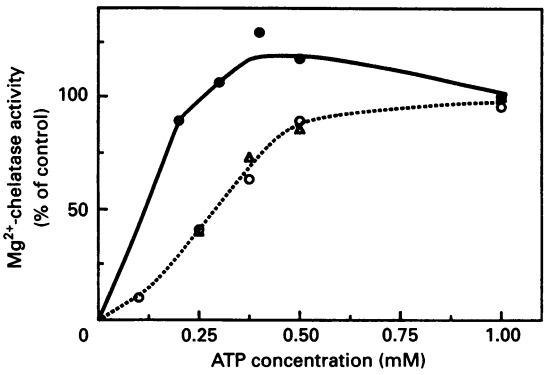

Figure 5 Separate effects of ATP concentration on activation and Mg^{2+} insertion

For all samples, 0.85 mg of LM/S protein was preincubated with ATP in 0.1 ml. After 6 min at 30 °C, the samples were diluted to a volume of 0.5 ml with buffer containing ATP and Deutero. The activities were measured by the continuous assay and calculated from the slopes of the linear portions or product-accumulation curves. In the first set of samples (△), the ATP concentrations were identical in the preincubation and the assay. In the second set (○), the ATP concentrations were varied in the preincubation as indicated, and all the assays were performed at a final concentration of 0.5 mM ATP. In the third set (●), the ATP concentration in all the preincubations was 1 mM, but in the assay the ATP concentrations were varied as indicated. All preincubations and assays in each set of samples contained the standard concentration of the regenerating system. Each set of data was generated on different days, using frozen portions of the same LM/S preparation. On each day the same control (preincubation at 1 mM ATP and assay at 1 mM ATP) activity was measured (147 units/incubation) and defined as 100%. Each data set was normalized to this activity; the variation in control activities on different days was at most 15%.

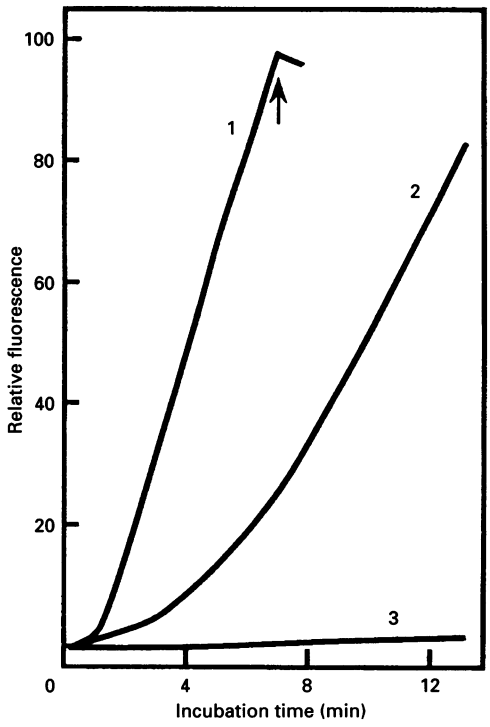

Figure 6 Use of the ATP analogue, ATP[S], to activate Mg^{2+}-chelatase

In line 1, LM/S (0.8 mg of protein) was preincubated for 6 min at 30 °C in 100 μl of buffer containing 1 mM ATP[S], the sample was diluted with buffer containing Deutero and ATP; the final concentrations of ATP[S], ATP and Deutero were 0.2 mM, 2 mM and 9 μM respectively. Glucose (50 mM final concentration) and hexokinase (10 units) were added at the point indicated on the figure. In line 2, the same amount of LM/S was assayed without any preincubation and the concentrations of substrates were the same as in line 1. In line 3, LM/S was preincubated under conditions identical with those for line 1, but was diluted with buffer containing ATP[S] (final concentration, 2 mM) instead of ATP. For all samples the ATP-regenerating system was omitted. The specific activity of the sample in line 1 was 207 units/mg.

ATP analogues

If ATP plays a different role in each of the two steps, it is possible that an ATP analogue might substitute for ATP in one step but not the other. Therefore we tested a number of ATP analogues as substrates for the first step. Testing these analogues directly for their ability to support the second step was not possible as this would involve removing ATP after the preincubation, which is not feasible with our present experimental system. We have previously shown that the non-hydrolysable ATP analogue, AMPPCP, could not support Mg^{2+}-chelation in intact cucumber chloroplasts [13]. Samples preincubated with AMPPCP (2.0 mM) and subsequently diluted and assayed in the presence of Deutero and 1.0 mM ATP (and 0.5 mM AMPPCP) were inactive (results not shown). Samples preincubated with ATP (2.0 mM) and subsequently diluted and assayed in the presence of Deutero and 1.0 mM AMPPCP (and 1.5 mM ATP) had only 62% of the activity of a control without AMPPCP (results not shown). Thus this analogue could not support the activation step and was inhibitory to the Mg^{2+}-insertion step. Samples preincubated with the nucleotide triphosphates (GTP, UTP or CTP) at 2.0 mM and subsequently diluted and assayed in the presence of 1.0 mM ATP (and 0.5 mM nucleotide triphosphate) were inactive compared with a control preincubated with ATP (results not shown). Thus these nucleotides also could not support the first step.

ATP[S] is a substrate that can be used by many ATP-dependent enzymes [18], and has gained wide use as a substrate for protein kinases. Protein kinases can phosphorylate a protein with ATP[S], but the thiophosphate transferred is often resistant to removal by phosphoprotein phosphatases [18,19]. When LM/S was preincubated in the presence of 1.0 mM ATP[S] and then diluted and assayed at 2.0 mM ATP[S], no activity was measured (Figure 6, line 3). If the preincubation contained ATP[S] and the dilution buffer contained ATP (2.0 mM final concentration) instead of ATP[S], normal Mg^{2+}-chelatase activity was measured (line 1); Mg^{2+} chelation stopped as soon as the ATP trap (hexokinase/glucose) was added. The activity profile showed only a small lag (less than 1 min) before the onset of linear activity. This is in comparison with the 6–8 min lag observed when a sample did not undergo a preincubation, but was assayed in the presence of ATP (line 2). Thus preincubation with ATP[S] had allowed activation to take place.

There was no significant difference between ATP and ATP[S] as substrates in the first step. Table 3 shows that LM/S preincubated at either 1 mM ATP[S] or 1 mM ATP, then assayed at 1 mM ATP, had almost the same activity. When the LM/S was chilled on ice for 30 min after activation, both the samples preincubated with ATP and ATP[S] were inactivated. If the LM/S was left at room temperature for 30 min after activation, only the sample preincubated with ATP[S] retained its full activity; the sample preincubated with ATP (and no regenerating system) lost activity. As we have observed ATPase activity in these preparations (unpublished work), the high activity in the sample preincubated with ATP[S] then left at room temperature (compared with the same treatment with ATP) may be due to a preferential depletion of ATP relative to ATP[S] by ATPases present in the sample [19]. An ATP-regenerating system is usually included in our assays, but in this case it had to be omitted to

Table 3 Does activation with ATP[S] prevent the loss of the activated state on chilling?

LM/S protein (0.82 mg) was preincubated in 0.1 ml of buffer for 6 min at 30 °C in the presence of either 1 mM ATP or 1 mM ATP[S]. After the preincubation, the sample received one of the following treatments: the control was diluted to 1.0 ml and assayed at 1 mM ATP (or 1 mM ATP and 0.1 mM ATP[S], when the preincubation contained ATP[S]); a sample was chilled on ice for 30 min, then diluted with buffer and assayed as for the control; the other sample was incubated at room temperature for 30 min, then assayed as for the control. None of the incubations contained the ATP-regenerating system. Both the control and the room temperature-treated sample were diluted with 0.8 ml of 30 °C buffer + 0.1 ml of ice-cold buffer; the chilled sample was diluted with 0.9 ml 30 °C buffer. Mg^{2+}-chelatase activity was measured using the continuous assay.

	Mg^{2+}-chelatase activity (units/incubation)	
Sample treatment	ATP	ATP[S]
Control	150	177
Chilled	29	46
Room temperature	65	184

prevent the regeneration of ATP in the ATP[S] preincubation. Thus ATP[S] is a good substrate for the first step, and can substitute for ATP in protecting the LM/S from the previously reported room temperature inactivation in the absence of ATP [12]. However, ATP[S] was completely unable to sustain activity in the second step and could not protect the activated LM/S from inactivation by the cold. The same pattern of results was obtained in a duplicate experiment (results not shown), where the dilution factor from preincubation to incubation was 5-fold rather than 10-fold as shown in Table 3.

DISCUSSION

Several lines of evidence point to enzymic Mg^{2+} chelation proceeding by a two-step reaction. The first indication was the several minute lag period before Mg^{2+} chelation started. As this lag could be eliminated by preincubation with ATP in the absence of porphyrin substrate, it indicated that an ATP-dependent process preceded Mg^{2+} insertion [12]. We have now shown that, in order to achieve optimal activity, there must be a high concentration of both LM and S proteins present during the activation process; as this high protein concentration is not required for Mg^{2+} chelation, we can divide the reaction into two steps with distinct protein concentration requirements. Similarly, the ATP requirement can be divided into two separate requirements: the activation is optimal at about 1 mM ATP (EC_{50}, 0.3 mM), whereas Mg^{2+} chelation proper is supported at lower ATP concentrations (0.3 mM ATP is almost optimal and the EC_{50} is less than 0.2 mM). These data again suggest that there is a two-step process, with each step having its own response to various ATP concentrations. A further clear distinction between the activation and chelation steps was shown by probing the nature of the ATP needed for activity. Activation can take place if ATP is replaced by the analogue ATP[S]; however, ATP[S] is completely unable to support Mg^{2+} insertion.

Therefore we suggest that Mg^{2+} chelation is a two-step process, consisting of activation followed by catalysis (Mg^{2+} insertion), with each step having individual requirements for proteins and substrates. At this point the exact mechanisms of the two steps have not been determined. However, we can now characterize the properties of these steps more fully.

The activation step requires both LM and S fractions to be present (Table 1). As the reaction only takes place when a threshold concentration of protein is present, and proceeds maximally when the LM/S is preincubated at a higher protein concentration (Figure 2), we assume that the components interact with each other during the activation step. Activation also requires the presence of millimolar levels of hydrolysable ATP or ATP[S] (Figures 1, 5, 6 and Table 3).

A typical mode of ATP-dependent protein activation is protein phosphorylation mediated by a protein kinase. Protein kinases will often utilize the ATP analogue ATP[S] for phosphorylation [18], and, indeed, we have found that the activation step can take place with this substrate. However, it should be noted that ATP[S] can be used by ATP-dependent enzymes other than protein kinases, and the fact that Mg^{2+}-chelatase can use this substrate cannot be considered proof of their involvement [18]. As the chloroplast has at least 40 proteins that are phosphorylated by protein kinases (most of these unidentified) [20], and as we do not know the molecular mass of the Mg^{2+}-chelatase components, the facile experiment of adding radiolabelled ATP to our assays and looking for phosphorylated proteins is not appropriate at this time. Another ATP-dependent activation process that takes place in chloroplasts is the activation of Rubisco by its activase. Although Rubisco activase cannot utilize ATP[S] as a substrate [21], possibly another activase-type enzyme exists in the chloroplast which can use this ATP analogue and is involved in the Mg^{2+}-chelatase reaction.

Thus far we are unable to conclude whether or not the activation is mediated by a protein kinase. That aside, the question remains what is the exact nature of the activation process? The cryoinactivation phenomenon (Figure 3 and Table 3) may provide a clue. Cryoinactivation has been reported for enzymes that are active as oligomers (e.g. lactate dehydrogenase [22]); no monomeric or dimeric enzyme has been reported to be subject to cryoinactivation [23]. If Mg^{2+}-chelatase is active only in an oligomeric form, the first step might involve the assembly of subunits. ATP may be required for this oligomerization as is the case for assembly mediated by chaperonins [24], or it may be required directly as an activator. A protein kinase could also participate in this oligomerization if the phosphorylation state of the subunits affects their association; protein phosphorylation promotes oligomerization in glycogen phosphorylase [25]. Many enzymes that are active in the multimeric state also exhibit lag times in their kinetics [26]. For example, lactate dehydrogenase has a several minute lag period which is due to the association of enzyme subunits into an active tetramer [27]. As with many allosteric enzymes, this lag period is reduced by preincubation with an activator.

In the second catalytic step, it is not possible to tell if both LM and S fractions are needed. The fact that this step does not require the high concentrations of protein needed for activation (Figure 2) might indicate that only one activated fraction is needed to catalyse Mg^{2+} insertion proper. In addition, we have preliminary evidence that, after activation, the S fraction alone is capable of catalysing the Mg^{2+} insertion (Table 2, treatment A). However, as the activity in activated S fraction is still synergistically increased on the addition of LM, this result must be interpreted with caution. Unfortunately, we routinely find measurable cross-contamination between LM and S fractions, and a clear answer to the question of how many proteins catalyse each step must await a better separation of the enzyme components.

We previously suggested that the reason why Mg^{2+}-chelatase, but not ferrochelatase, required ATP for catalysis was because of

an initial ATP-dependent activation preceding Mg^{2+} insertion, the implication being that metal-ion insertion itself did not require ATP. However, we now find that the second step (Mg^{2+} insertion) does need ATP (Figure 4). This ATP is needed at lower concentrations than for activation and cannot be replaced by ATP[S]. These data suggest that Mg^{2+}-chelatase may actually resemble cobaltochelatase in the vitamin B_{12}-biosynthetic pathway more than it resembles ferrochelatase. In an interesting recent paper, cobaltochelatase was purified from *Pseudomonas dentrificans* [28]. The enzyme consisted of two soluble components, one of which contained two different polypeptide subunits in a multimeric complex (possibly an octamer). ATP also was needed for catalysis and bound strongly to the complex, but not the other soluble component. As is the case for Mg^{2+} insertion, the role of ATP in cobalt ion chelation has not been defined, nor was there any indication of an activation step or a lag period in the kinetics.

Since Castelfranco's group reported that Mg^{2+} chelation was an ATP-dependent process, it has been speculated that, unlike the reaction catalysed by ferrochelatase, Mg^{2+} chelation is not a simple reaction requiring only porphyrin and metal ion substrates. Initially, the lack of a plastid-free system made it difficult to pinpoint a role for ATP in the reaction, and the mechanism of Mg^{2+} chelation remained something of a black box. However, using the *in vitro* plastid-free system, we have now made some specific observations as to how Mg^{2+} chelation is achieved and as to how ATP is involved. Most significant perhaps is the discovery that Mg^{2+} chelation requires a multicomponent enzyme system which catalyses Mg^{2+} insertion in a distinct two-step process. We have reported some interesting characteristics of both the steps that should give clues as to the true mechanism of this enzyme. Before a reasonable model can be proposed for the reaction mechanism, it is essential to establish how many components make up the enzyme system and to obtain fractions of these components with minimal cross-contamination. This will involve solubilization of the activity which is currently in progress in our laboratory.

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